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RESEARCH ARTICLE

Fibroblasts from long-lived bird species are resistant to multiple forms of stress

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SUMMARY

Evolutionary senescence theory postulates that aging results from the declining force of natural selection with increasing chronological age. A goal of comparative studies in the biology of aging is to identify genetic and biochemical mechanism(s) driving species-specific differences in the aging process that are the end product of life history trade-offs. We hypothesized that cells from long-lived bird species are more resistant to stress agents than are cells from short-lived species, and that cells from birds are more resistant to stress than are cells from relatively short-lived mammals of similar size. We tested primary fibroblast cultures from 35 species of free-living birds for their resistance to multiple forms of cellular stress and found that cell lines from longer-lived species were resistant to death caused by cadmium (R^2=0.27, P=0.002), paraquat (R^2=0.13, P=0.03), hydrogen peroxide (R^2=0.09, P=0.07) and methyl methanesulfonate (R^2=0.13, P=0.03), as well as to the metabolic inhibition seen in low-glucose medium (R^2=0.37, P<0.01). They did not differ in their resistance to UV radiation, or to thapsigargin or tunicamycin, inducers of the unfolded protein response. These results were largely consistent even after accounting for the influence of body mass and phylogeny. Cell lines from longer-lived bird species also proliferate more rapidly than cells from short-lived birds, although there was no relationship between proliferation and stress resistance. Finally, avian fibroblasts were significantly more resistant than rodent fibroblasts to each of the tested stressors. These results support the idea that cellular resistance to injury may be an important contributor to the evolution of slow aging and long lifespan among bird species, and may contribute to the relatively long lifespan of birds compared with rodents of the same body size.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/11/1902/DC1

Key words: longevity, evolution, fibroblast, stress, oxidation.

INTRODUCTION

Evolutionary senescence theory postulates that aging results from the declining force of natural selection with increasing chronological age (Medawar and Wolstenhilme, 1955; Charlesworth, 1993). A goal of comparative studies in the biology of aging is to identify genetic and biochemical mechanism(s) driving species-specific differences in the aging process that are the end product of life history trade-offs (Kirkwood, 2002). Although cellular architecture and signaling pathways are highly conserved across multiple taxonomic levels, there are striking disparities in the pace of aging [see recent reviews by Austad (Austad, 2010), Miller et al. (Miller et al., 2011) and Ricklefs (Ricklefs, 2010)]. For example, the kidney, skin and eyes of mammals are composed of the same cell types and share similar ultrastructural organization, but the range of maximum lifespans, a useful surrogate for aging, varies by as much as 40-fold. It is unclear whether those factors that contribute to the evolution of long lifespan among bird species overlap with those seen within mammals.

Primary fibroblast cell cultures vary in ways that correlate with species-specific longevity among mammals. For example, the resistance of fibroblasts to oxidative stress was positively correlated with species lifespan across several taxonomic orders (Kapahi et al., 1999), and significant differences in telomere biology and regulators of cell growth in rodents with disparate lifespans have also been reported (Seluanov et al., 2007; Seluanov et al., 2009; Seluanov et al., 2008). Harper et al. (Harper et al., 2007) has shown that fibroblasts from long-lived species of rodents and bats are also significantly more stress resistant than are cell lines from shorter-lived mammals (Harper et al., 2007; Salmon et al., 2008a) and a similar pattern of increased stress resistance was seen in cells from long-lived mutant Snell, Ames and growth hormone receptor knockout mice (Murakami et al., 2003; Salmon et al., 2005; Leiser et al., 2006). Hence, we hypothesized that fibroblasts from long-lived birds would also be more resistant to stress than cells from short-lived birds. In the case of mice, cells from long-lived stocks differ from control cells in DNA repair capacity (Salmon et al., 2008b), and signaling through the Nrf2 (Leiser and Miller, 2009) and MAP kinase pathways (Sun et al., 2009), although the extent to which these differences affect cells *in vivo* is unclear, as is whether differential signaling through these pathways is conserved among long-lived species.

Among higher level groupings, there are consistent taxon-specific differences in species lifespan. For example, birds typically live 1.5 times longer than mammals of the same size (Holmes and Martin, 2009), and a number of mouse-sized passerines (e.g. swallows and sparrows) can live as much as three to four times longer than mice. Birds achieve this remarkable longevity despite their exceptionally high metabolic rate coupled with the high energetic demands of

Table 1. Summary of bird fibroblast samples used in this study[a]

Taxonomic order	Taxonomic authority	Common name	Maximum lifespan (years)	Body mass (g)	Number of species	Number of individuals
Anseriformes	Wagler 1831	Ducks, geese	20–42	340–3650	7	19
Apodiformes	Peters 1940	Hummingbirds	9	3	1	9
Charadriformes	Huxley 1867	Shorebirds	11–32	90–520	3	8
Columbiformes	Latham 1790	Doves	31	120	1	15
Galliformes	Temminck 1820	Fowl (pheasant)	27	1100	1	1
Piciformes	Meyer and Wolf 1810	Woodpeckers	12	26	1	3
Passeriformes	Linneaus 1758	Songbirds	5–23	10–111	20	55
Struthioniformes	Latham 1790	Ratites (ostrich)	50	111,000	1	3

[a]A detailed species list is available in the supplementary material Table S1.

flight (and the resultant high lifetime energy expenditure), an elevated core body temperature and elevated levels of circulating glucose and lipids (Holmes and Ottinger, 2003). Because of this consistent difference in lifespan in birds *versus* mammals, we also hypothesized that bird cells would be more resistant to stress than mammal cells.

There have been few studies of the stress resistance properties of bird cells. One report noted that renal epithelial cells from three bird species (budgerigars, starlings and canaries) were significantly more resistant to oxidative stress and DNA damaging agents than was a mouse cell line tested in parallel (Ogburn et al., 1998), and a second report from the same group found cells from a short-lived bird (Japanese quail) to be less stress resistant than cells derived from a longer-lived species (budgerigar) (Ogburn et al., 2001). We have also recently shown that cell lines from almost all bird species were more resistant than mouse or rat cells to the lethal effects of cadmium (Cd), hydrogen peroxide (H_2O_2), paraquat and methylmethane sulfonate (MMS), a DNA alkylating agent (Miller et al., 2011). Here we confirm this result using additional avian and mammalian species. More significantly, we now show that increased longevity among birds is associated with higher levels of cellular resistance to Cd, H_2O_2, paraquat and MMS using dermal fibroblasts derived from more than 30 species of birds collected directly from the wild, and therefore not subject to the undue selection pressures associated with the artificial conditions of domestic life (Miller et al., 2002; Harper et al., 2006). In contrast, there was no relationship between cellular resistance and species lifespan for the lethal effects of UV irradiation or either of two inducers of the unfolded protein response, i.e. tunicamycin and thapsigargin.

Finally, within mammals there is an association between the rate of cellular proliferation and species lifespan in that cells from long-lived species readily enter replicative senescence or exhibit extremely slow rates of continuous growth whereas cells from short-lived species show rapid, continuous growth (Seluanov et al., 2008). These differences in cellular growth properties are thought to underlie differences in the incidence of neoplastic disease among mammals. We expected the cells from long-lived birds to proliferate more slowly than those from short-lived species. However, in contrast to our expectation, we show that cells from long-lived species proliferate more rapidly than cells from shorter-lived species, and that this relationship remains significant after adjusting for species body mass.

MATERIALS AND METHODS

Establishment of bird cell lines

For most species, cell lines were established from the skin of adult free-living birds caught using mist nests in and around north-central Ohio during the summer months. Skin samples from ducks and geese were taken from birds collected by hunters during October, and ostrich skin was collected from young adult birds at an abattoir in Illinois. All samples were obtained in accordance with USFWS and Ohio collecting permits. Immediately after birds were killed, feathers were plucked from an area on the wing or abdomen, the exposed skin was washed with anti-microbial soap and a $5\times5\,mm^2$ biopsy was excised and placed into cold 'complete' cell culture medium [Dulbecco's modified Eagle medium (DMEM), high-glucose variant ($4.5\,mg\,ml^{-1}$), with sodium pyruvate ($110\,mg\,ml^{-1}$), supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum and antibiotics ($100\,U\,ml^{-1}$ penicillin/streptomycin) containing $10\,mmol\,l^{-1}$ HEPES]. Skin samples were shipped on ice overnight to the University of Michigan for further processing. In total, we established cell lines from 115 individuals of 35 species spanning eight avian orders (Table 1). Supplementary material Table S1 provides a complete species list with additional details.

Fibroblast cultures from all biopsies were established as previously described after an overnight digestion in collagenase B (Murakami et al., 2003; Salmon et al., 2005; Harper et al., 2007) and cells were harvested by trypsinization (0.25%) for subculturing when they reached 90% confluence. Because of differences in the rate of initial cell outgrowth among species, the initial passage (P0) took place 6 to 14 days after establishing the original culture. Every 3–4 days, 75% of the medium was replaced with fresh complete medium, and cells were subcultured at day 7–10 into new $75\,cm^2$ flasks (P1) at a density of $10^4\,cells\,cm^{-2}$. After an additional 7–10 days of proliferation, cells were harvested and cryopreserved at $10^6\,cells\,ml^{-1}$ in DMEM supplemented with 40% fetal bovine serum and dimethylsulfoxide (DMSO) at a final concentration of 20% and stored in liquid N_2 for up to 12 months prior to assessment of stress resistance and the rate of cellular proliferation (see below).

Initially, cells from 34 individuals were grown in a humidified incubator with 5% CO_2 in room air. Because there is evidence that exposure to 21% O_2 can lead to early growth crisis relative to cells grown at lower oxygen tension (Busuttil et al., 2003; Maynard and Miller, 2006; Parrinello et al., 2003), we elected to grow the remaining 81 cell lines in an incubator maintained at 3% O_2 prior to cryopreservation. To determine whether initial O_2 concentration significantly affects cellular stress resistance, fresh skin biopsies from 14 individuals of eight species were divided into halves, expanded in either 21 or 3% O_2, then tested for their resistance to Cd, H_2O_2, paraquat, MMS, ultraviolet (UV) radiation, tunicamycin and thapsigargin; there was no significant effect of initial O_2 tension on the mean LD_{50} among replicates (ANCOVA, $P>0.08$ for all; supplementary material Table S2). Likewise, because avian core temperature is typically closer to 40°C, individual cell lines (N=12 individuals of seven species) were expanded and tested at both 37 and 40°C to determine whether temperature significantly altered their stress resistance properties; there was no significant effect of

temperature on resistance to Cd, UV, paraquat or MMS (ANCOVA, $P>0.3$; data not shown).

All stress tests were conducted using cells grown from cryopreserved aliquots (P1) in 3% O_2 at 37°C for 7–10 days prior to harvesting for the stress assay. Cells (P2) were thawed and placed in 10 ml of complete medium in 75 cm^2 flasks at 5×10^5 cells $flask^{-1}$ (6.7×10^3 cells cm^{-2}), fed on day 3 (75% replacement) and then subcultured on day 7 into 175 cm^2 flasks (P3) at 10^5 cells cm^{-2}. After 1 week of further proliferation, these P4 cells were harvested and used for stress testing. Because of low numbers, cells from four individual birds (one each from chipping sparrow, cowbird, pheasant and northern shoveler) needed to be expanded for an additional passage and were tested at P5.

Assessment of fibroblast resistance to lethal stress

We tested cellular resistance to multiple agents using between one and 15 individual cell lines for a given species. In cases where sufficient numbers were available, the remaining P4 cells were used to assess cellular proliferation rate as described below. Because of the large number of lines, we were unable to assay stress resistance in all of them at once. Instead, individual cell lines were assayed on one of 15 test dates over the course of 6 months, with the majority of cell lines (74/101 individual birds) tested over the course of six consecutive assay dates. Typically, cells from 12 individuals were assayed in parallel.

For testing, 3×10^4 cells in 100 µl of complete medium were seeded into 96 well microtiter plates, allowed to attach overnight (24 h) and then exposed to DMEM lacking serum and sodium pyruvate but containing 2% bovine serum albumin for an additional 24 h. Immediately afterwards, cells were exposed to graded doses of Cd, H_2O_2, paraquat, MMS, tunicamycin or thapsigargin for 6 h, or to graded doses of UV (Harper et al., 2007; Murakami et al., 2003; Salmon et al., 2008a; Salmon et al., 2005). Immediately afterwards, cells were washed once with PBS, and incubated in serum-free medium for an additional 18 h. Cell survival was evaluated using reduction of the extracellular tetrazolium dye WST-1 to its colored formazan product; visual inspection of the plates revealed extensive cell death at high concentrations of stress agents. Control cells, incubated without any stress agent, were included in each set of assays, and we found no significant difference among the test dates for these unstressed cells using WST-1 reduction as a measure of cell number and viability (one-way ANOVA, data not shown). All tests were carried out at 37°C in a humidified incubator with 5% CO_2 in 3% O_2.

Response to glucose withdrawal

Culture in medium with very low graded glucose levels impairs the ability of fibroblasts to reduce the extracellular tetrazolium dye WST-1, but does not lead to cell death, and fibroblasts from Snell dwarf mice and long-lived rodents are resistant to these non-lethal effects of diminished glucose *in vitro* (Leiser et al., 2006; Harper et al., 2007; Leiser and Miller, 2009). To assess the response of bird cells to low glucose culture conditions, P4 cells were plated at a density of 3×10^5 ml^{-1} in DMEM with 10% fetal bovine serum with 2% chicken serum and antibiotics. After an overnight incubation, cells were washed twice with PBS (37°C) and incubated in DMEM containing a range of glucose concentrations, using glucose-free DMEM supplemented with 10% dialyzed fetal bovine serum, antibiotics (100 U ml^{-1} penicillin/streptomycin) and graded amounts of supplemental glucose. Standard complete medium, containing glucose at 4.5 mg ml^{-1}, was used as a control and the quantitation of WST-1 reduction was determined 3 h later.

Cellular proliferation assay

Individual cell lines were seeded in duplicate wells on each of six replicate plates (i.e. 12 wells total) at a density of 3.2×10^3 cells in 96 well microtiter plates in 100 µl complete medium. At the end of days 1, 2, 3, 4, 5 and 9, one plate from each set of replicates was placed into a –80°C freezer and stored until all six replicates had been collected. Cell number was estimated using a fluorescence assay based on binding of Hoechst 33258 to DNA with the cell number in each well directly proportional to the intensity of the fluorescent signal. In total, cells from 48 individuals of 21 species were evaluated in two separate batches. To control for batch-to-batch variation, proliferation for each cell line was expressed as a ratio of the Hoechst fluorescence value for day 1 against the value on days 2–9; an increase in the value of this ratio was interpreted as a measure of cellular proliferation.

Data analysis

The concentration or dose needed to kill 50% of the cells (LD_{50}) was calculated using the FORECAST function in Excel for each of the lethal stressors; individual cell lines were tested in duplicate, with one microtiter plate per stressor. In the case of glucose, this value (ED_{50}) is reflective of a 50% reduction in cellular activity as indicated by the reduction of WST-1. Two data points, a downy woodpecker response to tunicamycin and a house wren response to MMS, had calculated LD_{50} values that were more than threefold higher than the LD_{50} for any other bird cell line tested; thus, these values were presumed to be the result of experimental error, and were removed as outliers prior to statistical testing.

Species mass as recorded in the AnAge database (http://genomics.senescence.info/species/) was used for birds and mammals. Maximum lifespan estimates for birds came from AnAge or were from bird band return records maintained by the United States Geological Survey (http://www.pwrc.usgs.gov/BBL/homepage/longvrec.htm); maximum lifespan estimates for mammals came from AnAge.

Statistical analyses were conducted using NCSS (Kaysville, UT, USA) or SAS (Cary, NC, USA) except for the analysis of phylogenetically independent contrasts (PICs). We used simple linear regression to examine the relationship between species maximum lifespan and the mean LD_{50} for each of the seven agents, and to examine the relationship between cell proliferation, species body mass and lifespan. With the exception of a few species that were represented by a single individual, the mean LD_{50} generated from multiple individuals within a species was used in the regression analysis, using one mean LD_{50} value for each species.

In addition, we used a multi-level regression that assumed the test species were randomly selected from the entire spectrum of extant species, and consequently treated these data as clustered observations, with clusters of individuals being observed within species. To evaluate the relationship between LD_{50} and species lifespan, we used a linear mixed effects model with a random intercept describing variation between species. We also evaluated, in parallel, linear and quadratic fixed effects associated with species-specific lifespan, and models allowing heterogeneity of the residual variance, but these models did not improve the fit to the data and hence are not presented.

We also constructed models to determine whether the relationship between lifespan and stress resistance might simply reflect the well-known relationship between increased lifespan and body size (Austad, 2005; Austad and Fischer, 1991). Specifically, simple linear regression was used to examine the relationship between the residuals of each stress resistance measure (one value per species,

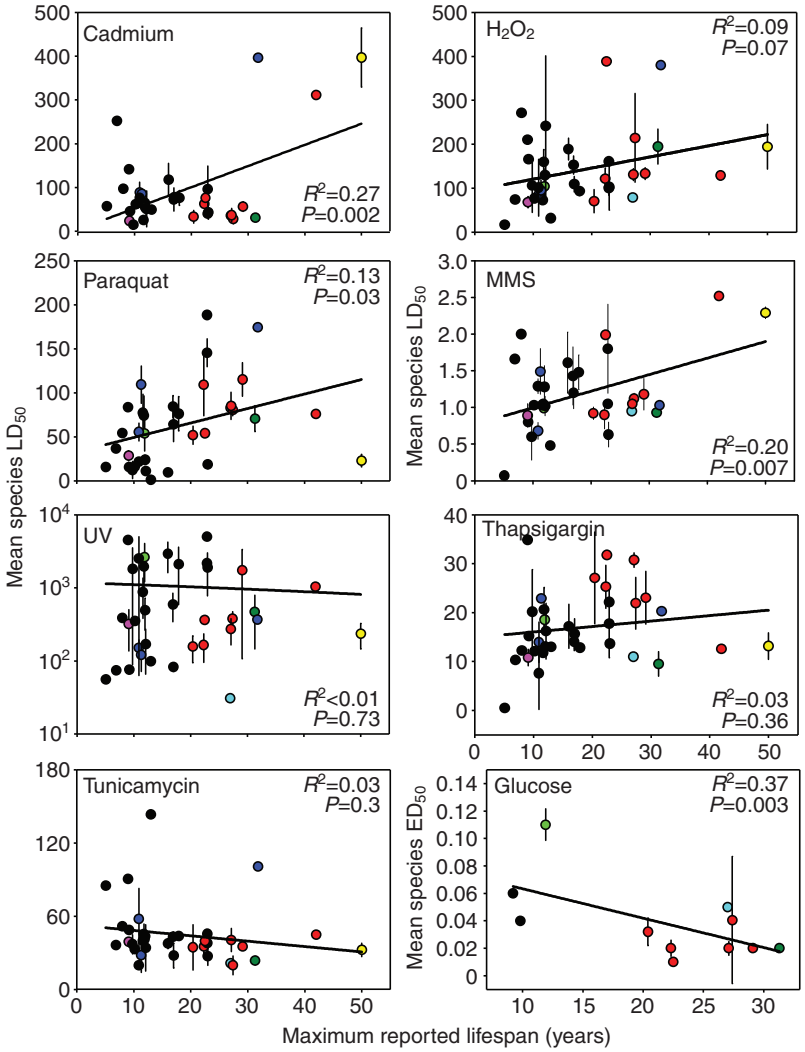

Fig. 1. The association between species maximum lifespan and the mean LD_{50} or ED_{50} value for each of the test species. Each color indicates all individual species from a given order. Red, Anseriformes; purple, Apodiformes; blue, Charadriformes; green, Columbiformes; turquoise, Galliformes; black, Passeriformes; light green, Piciformes; yellow, Struthioniformes. Number of species and independent samples represented by each order are given in Table 1. Error bars are ±s.e.m. The lines show the outcome of a least squares regression. Pearson R^2- and P-values reflect standard linear regression of maximum lifespan against mean LD_{50} or ED_{50} values for each species. Units are μM (cadmium, H_2O_2 and thapsigargin), mmol l^{-1} (MMS and paraquat), J m^{-2} (UV light), mg ml^{-1} (glucose) or μg ml^{-1} (tunicamycin).

after adjustment for species body mass) and the residuals of the estimated maximum lifespan (also after the adjustment for body mass). Similar adjustments were made for the cellular proliferation data.

We also employed regressions based upon PICs as generated by Phenotypic Diversity Analysis Programs (Ives et al., 2007). Our phylogeny for the 35 test species was constructed in Mesquite (Maddison and Maddison, 2010), with branch lengths and topology based on Sibley and Ahlquist and others (Sibley and Ahlquist, 1990; Johnson and Sorenson, 1999; Klicka et al., 2000; Donne-Goussé et al., 2002; Yuri and Mindell, 2002; Barker et al., 2004; Reznick and Ricklefs, 2009). Diagnostic tests showed that contrasts were adequately standardized in all cases; hence no adjustment to branch lengths was made in our final model (Diaz-Uriarte and Garland, 1996; Garland et al., 1992). As for most other studies, we found that regressions based on PIC and conventional least squares were remarkably similar (Price, 1997; Ricklefs and Starck, 1996).

To compare the resistance of avian fibroblasts in this study with that of fibroblasts from mammals, we used the data on cell resistance of mammals from Harper et al. (Harper et al., 2007), as well as measures of cellular stress resistance for primary fibroblasts derived from 14 primate species, including human (R.A.M. and J.M.H., unpublished), with species body mass as a covariate in the final model.

RESULTS

Resistance of fibroblasts to lethal effects of cytotoxic agents and to culture in low glucose medium

Using simple linear regression, we saw a significant positive association between species maximum lifespan and cellular resistance to Cd, paraquat and MMS ($P \leq 0.05$ for all), whereas the regression for H_2O_2 showed a similar trend at P=0.07 (Fig. 1). Cell lines from longer-lived birds were also resistant to glucose deprivation, a result that is consistent with the resistance seen in rodent cells (Leiser et al., 2006; Harper et al., 2007). In contrast, there was no evidence for an association between lifespan and resistance to death induced by UV, thapsigargin or tunicamycin (Fig. 1).

Because there is no consensus on the optimal method of evaluating associations between cellular properties and species lifespan, a variety of alternate statistical approaches were used to test the strength of the associations that we found (Table 2). A weighted regression method that adjusted for differences in the number of individual cell lines tested, whereby the weight of the species mean was assigned a value of $1/N$ with N equal to the number of individual cell lines, confirmed our impression of an association between maximum lifespan and resistance for Cd, paraquat and MMS ($P<0.04$ for each); the H_2O_2 data showed a similar, but insignificant, trend at P=0.07. Moreover, after standard $\log_{10}$ or square root transformation (as appropriate), the associations for paraquat, H_2O_2 and MMS remained significant

Table 2. Comparison of analysis methods for association between bird species lifespan and stress resistance

	Simple linear regression		Weighted regression		LD_{50} residual × Lifespan residual		Multi-level regression	Phylogenetically independent contrasts	
Stressor	R^2	P	R^2	P	R^2	P	P	R^2	P
Cadmium	**0.27**	**0.002**	**0.24**	**0.003**	0.09	0.09	0.06	**0.22**	**0.004**
H_2O_2	0.09	0.07	**0.13**	**0.03**	0.09	0.08	**0.015**	0.01	0.54
Paraquat	**0.13**	**0.03**	0.1	0.07	**0.29**	**<0.001**	0.10	**0.14**	**0.03**
MMS	**0.13**	**0.03**	**0.17**	**0.03**	0.09	0.08	**0.048**	0.04	0.24
UV	<0.01	0.73	0.01	0.61	<0.01	>0.9	0.97	**0.11**	**0.05**
Thapsigargin	0.03	0.36	0.01	0.51	0.06	0.15	0.48	0.002	0.8
Tunicamycin	0.03	0.3	0.05	0.19	0.03	0.36	0.23	<0.01	>0.9

MMS, methylmethane sulfonate.
Bold type indicates significance at $P<0.05$.

($P<0.05$ for all), with a similar trend for the Cd data ($P=0.06$; data not shown). Regardless of approach, there was no indication of a relationship between avian lifespan and resistance to UV or inducers of the unfolded protein response.

Moreover, because of the well-documented trend towards longer lifespans among larger animals (Austad and Fischer, 1991), it has been suggested that regressions of longevity on physiological measures are meaningless without adjustment for species-specific body mass; in some cases, adjustment for species body mass does account for the statistical relationship between a cellular trait and lifespan (Lorenzini et al., 2005; Seluanov et al., 2007). Using our data (Table 2, third column) we find that a regression of the mass-adjusted lifespan residual against the mass-adjusted residual of the LD_{50} value results in a significant association for paraquat, with non-significant but suggestive associations for Cd, H_2O_2 and MMS ($P\leq0.09$ in each case). The values for UV, thapsigargin and tunicamycin remain largely unchanged. We also found that species body mass is associated with cellular resistance only for Cd and MMS ($P\leq0.02$); no relationship between species body mass and resistance to paraquat, H_2O_2, UV, glucose or either of the inducers of the unfolded protein response, tunicamycin and thapsigargin, was found ($P>0.5$ for all).

Each of the approaches described above uses the mean LD_{50} value for a given measure as representative of each species, even in cases when multiple cell lines were independently assessed. The fourth column of Table 2 shows the results of a different approach in which the stress resistance of each measure for an individual cell line was included in a two-level regression, similar to nested regression used in other contexts. Specifically, a linear mixed effects model was generated that partitioned the overall variation into among-species and within-species components, with the tabulated P-value representing the significance of the among-species term in the regression model. Using this approach, we found a significant relationship between lifespan and resistance to H_2O_2 and MMS ($P<0.05$), and suggestive, but insignificant ($P\leq0.09$), relationships for Cd and paraquat. Again, there is little support for any association between species lifespan and resistance to UV, thapsigargin or tunicamycin.

Finally, we evaluated the association between cellular stress resistance and species longevity after accounting for phylogenetic relatedness using the PIC method (Table 2) (Garland et al., 1992; Garland et al., 2005). Here, Cd and paraquat resistance showed a significant relationship with species lifespan whereas resistance to H_2O_2 and MMS did not. This method also suggested a significant association between lifespan and cellular resistance to UV. Consistent with the other analytical approaches, there was no association between lifespan and resistance to inducers of the unfolded protein response.

Cellular proliferation

We also evaluated the relationship between the rate of cellular proliferation and species lifespan. To adjust for differences in plating efficiency, we estimated proliferation rate as the ratio of cellular DNA at day 5 divided by the DNA content of replicate plates measured at day 1. Cells from longer-lived species proliferated more rapidly over the first 5 days of culture (Fig. 2A) with a similar relationship ($P=0.05$) also seen for plates evaluated at day 9. The relationship between the residuals of lifespan regressed against the residuals of the relative increase in cell number, after adjusting for body mass, indicates that even after this adjustment, the rate of

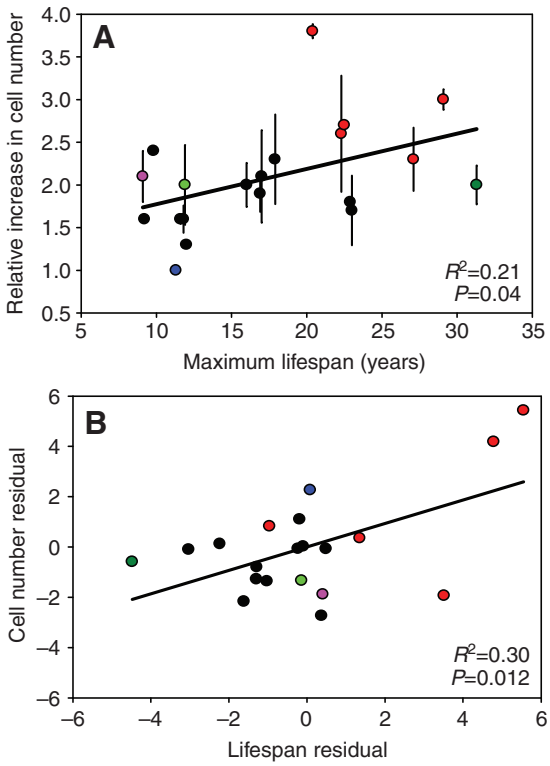

Fig. 2. Scatterplots showing the relationship between the relative increase in cell number 5 days after seeding and species maximum lifespan using either the (A) raw or (B) mass-adjusted data. Colors are as in Fig. 1 and error bars are ±s.e.m. The lines show values predicted by least squares regression. Pearson R^2- and P-values reflect standard linear regression of relative increase in cell number and the species maximum lifespan (A) or the body-mass-adjusted residuals of relative cell number *versus* species lifespan (B).

Table 3. Summary of ANCOVA results for bird *versus* rodent and primate cell line stress resistance

	Model 1			Model 2		
	Mass-adjusted mean LD_{50}			Mass-adjusted mean LD_{50}		
Stressor	Birds (35 spp.)	Rodents (9 spp.)	*P*	Birds (35 spp.)	Primates (14 spp.)	*P*
Cadmium (μM)	92.2	20.9	**0.01**	100.6	29.1	**0.01**
Paraquat (mM)	62.8	5.6	**0.001**	63.8	48.3	0.29
H_2O_2 (μM)	141.6	65.0	**0.01**	142.1	269.4	0.004[a]
MMS (mM)	1.19	0.35	**<0.001**	1.23	1.43	0.50
UV ($J\,m^{-2}$)	1046	123.4	**0.04**	1015	444	0.15
Glucose ($mg\,ml^{-1}$)	0.03	0.15	**<0.001**	0.03	0.11	**0.02**

Bold type indicates birds are significantly more resistant than the indicated group (rodents in model 1 and primates in model 2).
[a]The respective group of primates is significantly more resistant than birds.

cellular proliferation was still significantly associated with species lifespan on days 5 (Fig. 2B) and 9 (data not shown). There was no relationship between cellular proliferation and cellular stress resistance in the 21 species tested (data not shown).

Cellular stress resistance: comparisons of birds with mammals

We compared the body-mass-adjusted stress resistance profile of bird fibroblasts (N=11 species for glucose, 35 for all other stressors) with those values obtained from nine species of rodents, including one domestic and one wild-caught strain of *Mus musculus* (Table 3). We also compared data from birds with a set of unpublished data (R.A.M. and J.M.H.) for 14 species of primates (including humans), ranging in body mass from 0.25 to 62 kg and in maximum lifespan from 17 to 122 years (Table 3). In each case, ANCOVA models were constructed to evaluate the equality of slopes and y-intercept values of stress resistance *versus* lifespan between birds and rodents, or between birds and primates, with species body mass included as a covariate. Regardless of stressor, bird fibroblasts are significantly more resistant than rodent fibroblasts (Fig. 3). We also found that bird cells were significantly more resistant than primate cells to Cd and to the low-glucose condition, but there was no significant difference between avian and primate cell lines in their mean responses to paraquat, MMS or UV, and primate cells were significantly more resistant to H_2O_2 toxicity (Table 3). However, because the bird, rodent and primate cell lines were not tested in parallel, these data should be interpreted with caution.

DISCUSSION

Primary fibroblast cell lines isolated from 35 bird species spanning multiple taxonomic orders were used to examine the relationship between lifespan and resistance to cellular stress. Our working hypothesis was that cells from long-lived birds would be more stress resistant than cells from short-lived birds, as has been shown in

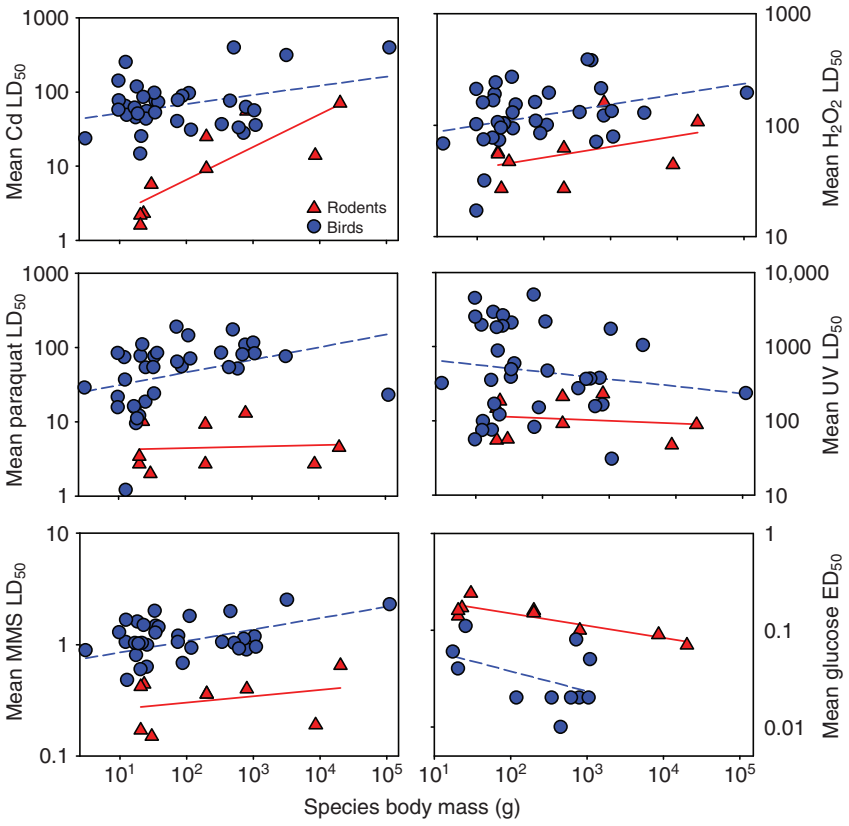

Fig. 3. Species mean LD_{50} or ED_{50} for each of the indicated agents plotted against species body mass. The lines show the outcome of a least squares regression for each of the indicated groups. Blue circles indicate birds, red triangles indicate rodents. Error bars have been omitted for clarity.

Table 4. Summary of results by analytical approach: lifespan *versus* stress resistance for birds

	Simple linear regression			Complex statistical models		
Stressor	Raw	Transformed	Weighted	Residual analysis	Multi-level regression	Phylogenetically independent contrasts
Cadmium	Y	T	Y	T	T	Y
Paraquat	Y	Y	T	Y	N	Y
H_2O_2	T	Y	Y	T	Y	N
MMS	Y	Y	Y	T	Y	N
UV	N	N	N	N	N	Y
Thapsigargin	N	N	N	N	N	N
Tunicamycin	N	N	N	N	N	N

Y, the association between resistance and longevity is at least marginally significant (two-tailed $P<0.05$); T, trend (two-tailed $P<0.1$); N, not significant (two-tailed $P>0.1$).

mammals (Kapahi et al., 1999; Murakami et al., 2003; Salmon et al., 2005; Maynard and Miller, 2006; Murakami, 2006; Harper et al., 2007; Salmon et al., 2008a). We found a significant association between species lifespan and the degree of resistance to lethal agents, as well as to the metabolic effects of glucose deprivation. Importantly, these results were supported using a variety of statistical methods differing in their power and complexity, as well as their underlying assumptions (Table 4). In general, increased resistance to Cd, paraquat, H_2O_2 and MMS was associated with increased maximum lifespan, although in some cases (e.g. mass-adjusted residual analysis) associations were only marginally insignificant ($0.1<P<0.05$) rather than meeting the two-tailed significance criterion of $P=0.05$. We also found that cell lines from longer-lived birds were more resistant to the effects of glucose deprivation than were cells from shorter-lived birds. An attempt to evaluate the effects of rotenone on cellular metabolism (Leiser and Miller, 2009) was unsuccessful in that bird cells as a whole were highly resistant to concentrations of rotenone that fully inhibited WST-1 conversion in mouse cell lines (data not shown). However, there was little to suggest a relationship between species maximal lifespan and resistance to UV, tunicamycin and thapsigargin.

A second working hypothesis was that, because birds are significantly longer-lived than similarly sized mammals, avian fibroblasts would be significantly more resistant to cytotoxic and metabolic stress compared with cells from mammals. Again, our hypothesis was supported, in that cells from birds were significantly more resistant than cells from rodents regardless of the stressor tested (Table 3). In each case the direction was as predicted, with effect sizes ranging from twofold to 11-fold. However, a comparison with primate fibroblasts gave a less consistent picture: bird cells were more resistant to Cd and glucose withdrawal, but more sensitive than primate cells to H_2O_2, with no significant differences in responses to the other stresses that we tested. Earlier studies of avian stress resistance also found that cells and tissues from birds were typically more resistant to oxidant damage and produced lower levels of reactive oxygen species (ROS) than cells from similarly sized mammals (Barja and Herrero, 1998; Ogburn et al., 1998; Herrero and Barja, 1999; Barja and Herrero, 2000; Ogburn et al., 2001; Pamplona et al., 2005; Lambert et al., 2007; Strecker et al., 2010).

Taken together, these data suggest that evolution of long-lived species may require augmentation of cellular pathways that regulate ROS-induced damage and damage to DNA. Both paraquat and H_2O_2 are well-known oxidative stressors, and there is evidence that the lethal effects of both Cd (Brennan and Schiestl, 1996; Figueiredo-Pereira et al., 1998; Shukla et al., 2000) and MMS (Mizumoto et al., 1993; Wilhelm et al., 1997) are at least partially mediated by oxidative stress; indeed, differences in the susceptibility of cell membranes to peroxidation are strongly associated with species lifespan (Faulks et al., 2006; Hulbert et al., 2006; Hulbert et al., 2007; Mitchell et al., 2007; Buttemer et al., 2008; Hulbert, 2008; Hulbert et al., 2008). Nevertheless, the mechanism(s) by which cells can augment resistance to multiple forms of lethal stress and metabolic perturbation are still unknown and are under active investigation (Lithgow and Miller, 2008; Williams et al., 2010), with the evaluation of co-evolutionary patterns likely to contribute to this developing story.

Tests of cellular proliferation rate in short-term (5 day) cultures suggested that longer-lived birds produced faster growing fibroblasts, irrespective of species body mass, and there was no consistent relationship between the relative increase in cell number and resistance to stress. In contrast to our data, Seluanov and colleagues (Seluanov et al., 2008) reported a significant negative relationship between the rate of cell growth and species lifespan in rodents. Interestingly, Seluanov et al. (Seluanov et al., 2008) also found that, as a consequence of significant differences in the degree of endogenous telomerase activity, cells from some species readily underwent cellular senescence whereas cells from other species did not. Unfortunately, our data from birds cannot be compared directly with these rodent data because the rodent study used a different measure of cell growth (population doublings per unit time) and excluded species whose cells readily underwent replicative senescence.

Although our data are from a collection of cell lines that is small relative to the totality of avian diversity, we note that at least one species of each of eight orders was included, representing approximately one-third of the currently recognized avian orders. Nevertheless, from Fig. 1 it is clear that individual species may exist as outliers that mask otherwise significant results (Type II error). For example, individual species that are exceptionally long-lived but are also particularly sensitive to given stressor can obscure a relationship that may be strong in a larger, and more representative, group of species. In addition, there is the possibility that some species have exceptionally high (or low) resistance to one or more forms of stress that is a reflection of their unique evolutionary history and is not an evolved trait *per se*. We note that we did include an adjustment for the degree of phylogenetic independence among the species being evaluated in our analyses (Garland et al., 2005), although it has been argued that adjustment for phylogeny is necessary only when there is a demonstrable effect of phylogenetic relatedness (Abouheif, 1999; Blomberg et al., 2003; Freckleton et al., 2002).

It is not yet clear whether long lifespan among species depends on the same, uniform set of cellular and molecular changes among clades, or instead reflects idiosyncratic processes for each long-lived

lineage, or some combination that allows a species to adapt to local conditions within clade-specific developmental constraints. Our studies of fibroblasts from rodents (Harper et al., 2007; Salmon et al., 2008a), and now from birds, suggest that cellular resistance to multiple forms of oxidative and DNA damage may be an important element in the evolution of increased longevity in multiple clades, but leaves open the possibility that there are cellular traits associated with slow aging and long lifespan in some, but not all, lineages. Dissection of the molecular pathways involved in interspecific differences in stress resistance may help us to deconstruct this aspect of comparative cell biology.

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